GENERATION AND CHARACTERIZATION OF DIABETIC RAT MODEL WITH DETECTION OF ADULT CARDIAC FIBROSIS

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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TO WHOM IT MAY CONCERN

This is to certify that **Sneha Roy**, a M.Sc. student of the Department of Biotechnology, Sister Nivedita University, Kolkata has successfully completed her dissertation research work in my research laboratory for the duration of about five months (**02/01/2025 to 30/05/2025**). During her tenure she got the opportunity to work on several molecular and cell biology techniques including but not limited to generation of diabetic rat model (*in vivo*), immunocytochemistry/immunohistochemistry, protein/RNA isolation, concentration measurements (spectroscopy), Real time PCR, Western blot, Histochemical staining (H&E, Masson's trichrome, Van Gieson's) and imaging (Fluorescence microscopy).

In closing, I certify that Sneha is a very punctual, obedient, inquisitive, dedicated, hardworking and sincere in nature. She is very attentive and regular and tries to give her best to assigned tasks. Apart from being a hard worker and sincere student, she has a habit of keeping herself up to date in relevant literature. I wish her success for all future endeavors in her life and career.

Best Regards,

Dated: June 02, 2025

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**Generation and characterization of diabetic rat model with detection of adult cardiac fibrosis**" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from January 2025 to May 2025 under the supervision of Dr. Santanu Chakraborty Department of Life Sciences.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

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Sneha Roy
06.06.2025

(Signature of the students with Date)

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ABBREVIATIONS: -

DM- Diabetes mellitus

STZ- Streptozotocin

TGF-β- Transforming Growth Factor-β

TGF-β1- Transforming Growth Factor-β

TNF-α- Tumor Necrosis Factor-α

IL-6- Interleukin-6

MCP-1- Monocyte Chemoattractant Protein-1

MMP9- Matrix Metalloproteinase 9

β-ACTIN- Actin beta

α-SMA- Alpha smooth muscle actin

ECV- External Electrical Cardioversion

DCM- Diabetic Cardiomyopathy

ECM- Extracellular matrix

CVF- Collagen Volume Fraction

RT-PCR- Reverse Transcription Polymerase Chain Reaction

PCR- Polymerase Chain Reaction

qPCR- Quantitative Polymerase Chain Reaction

H&E- Hematoxylin and Eosin

ELISA- Enzyme Linked Immunosorbent Assay

RAAS- Renin- Angiotensin- Aldosterone System

CTGF- Connective Tissue Growth Factor

MRI- Magnetic Resonance Imaging

ACE INHIBITORS- Angiotensin Converting Enzyme Inhibitors

ARBs- Angiotensin II Receptor Blockers

IAEC- Institutional Animal Ethics Committee

CPCSEA- Committee for the Purpose of control and Supervision of Experiments on Animals

I.P- Intraperitoneal Injection

PBS- Phosphate Buffered Saline

PFA- Paraformaldehyde

BMI- Body Mass Index

MESA- Multi-Ethnic Study of Atherosclerosis

PBST- Phosphate-Buffered Saline with Tween 20

DAPI- 4’6- diamidino-2-phenylindole

EDTA- Ethylenediaminetetraacetic acid

DOC- Sodium deoxycholate

SDS- Sodium Dodecyl Sulfate

ABSTRACT: -

Diabetes mellitus (DM) is a chronic metabolic disorder that significantly increases the risk of cardiovascular complications, including cardiac fibrosis—a major factor to induce diabetic cardiomyopathy. This study aimed to generate and characterize a reliable diabetic rat model to investigate the onset and progression of adult cardiac fibrosis associated with diabetes. Male Wistar rats were administered streptozotocin (STZ) intraperitoneally to induce diabetes. Diabetic status was confirmed through sustained hyperglycaemia and characteristic physiological markers. Over a specified duration, structural changes were assessed using histopathological analysis, and molecular assays to detect fibrosis-related markers such as collagen deposition and α-SMA expression. The results demonstrated a significant increase in fibrotic tissue accumulation in diabetic rats compared to controls, along with functional impairments consistent with diabetic cardiomyopathy. This model effectively replicates the pathological features of diabetes-induced cardiac fibrosis and provides a valuable platform for studying underlying mechanisms and evaluating potential therapeutic interventions.

KEYWORDS: -

Cardiac fibrosis, cardiomyopathy, diabetes mellitus, diabetic, streptozotocin

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INTRODUCTION: -

Diabetes mellitus (DM) is a growing global health concern, characterized by chronic hyperglycaemia due to impaired insulin secretion, insulin resistance, or both. Persistent hyperglycaemia triggers a cascade of metabolic and molecular disturbances, eventually leading to severe complications, including diabetic cardiomyopathy (DCM). DCM is a distinct clinical entity that involves myocardial remodelling, left ventricular dysfunction, and increased interstitial fibrosis, independent of hypertension or coronary artery disease [1]. Research indicates that obesity is strongly associated with cardiac fibrosis. A study published in the Journal of Cardiovascular Magnetic Resonance found that in obese adults, higher ECV% was associated with increased hospitalization for heart failure and death. For every 5% increase in ECV, the hazard ratios for heart failure hospitalization and death were 1.92 and 2.50, respectively [4]. One of the most critical pathological features of DCM is cardiac fibrosis, which involves the accumulation of extracellular matrix (ECM) proteins such as collagen I and III. This fibrotic remodelling stiffens the myocardium, impairs contractility, and promotes arrhythmogenesis, thereby contributing to heart failure. Understanding the mechanisms and progression of cardiac fibrosis in diabetes is vital for developing effective interventions [2]. To model this condition in vivo, the streptozotocin (STZ)-induced diabetic rat model is commonly employed due to its ability to selectively destroy pancreatic beta cells, mimicking type 1 diabetes mellitus. In this project, adult male Wistar rats will be used. Diabetes will be induced through a single dose of intraperitoneal injection of STZ (typically at 35 mg/kg body weight). Following STZ administration, blood glucose levels will be monitored, and rats with glucose levels above 250 mg/dL will be considered diabetic [3]. Over a defined period (usually 4 to 12 weeks), diabetic rats will undergo metabolic monitoring (e.g., blood glucose, body weight, food, and water intake). At the study endpoint, hearts will be harvested and examined for fibrotic changes using the following methods:

Histological analysis: Hematoxylin and Eosin (H&E) staining and Masson's trichrome staining to assess myocardial structure and collagen deposition.

Molecular assays: RT-PCR and Western blotting to quantify expression of fibrosis-related genes (e.g., TGF-β1, collagen I, fibronectin).

Biochemical analysis: Hydroxyproline assay for quantification of total collagen content [3].

The generated model and its characterization will contribute to a better understanding of diabetes-induced cardiac remodelling. It will also serve as a platform for testing anti-fibrotic drugs or genetic interventions. A systematic review and meta-analysis encompassing 32 studies with 5,053 participants found that individuals with diabetes, predominantly type 2, exhibited a higher degree of myocardial fibrosis compared to non-diabetic controls. Specifically, the collagen volume fraction (CVF) was on average 5.8% higher, and the extracellular volume fraction (ECV%) was 2.1% higher in diabetic patients. These findings suggest a significant association between diabetes and increased myocardial fibrosis [5].

In the context of obesity, studies have demonstrated a relationship between higher body mass index (BMI) and increased myocardial fibrosis. For instance, the Multi-Ethnic Study of Atherosclerosis (MESA) observed that individuals with higher BMI categories had elevated ECV%, a marker of myocardial fibrosis [6].

Additionally, in a study focusing on obese adults, diffuse myocardial fibrosis quantified by ECV was associated with subsequent hospitalization for heart failure and death. Specifically, for every 5% increase in ECV, there was a 92% increase in the risk of hospitalization for heart failure and a 150% increase in the risk of death while individual studies have examined the effects of diabetes and obesity on myocardial fibrosis separately, the combined impact of these conditions is less well-defined. However, given that both diabetes and obesity independently contribute to increased myocardial fibrosis, it is plausible that individuals with both conditions may experience additive or even synergistic effects, leading to more pronounced fibrotic changes in the heart [4].

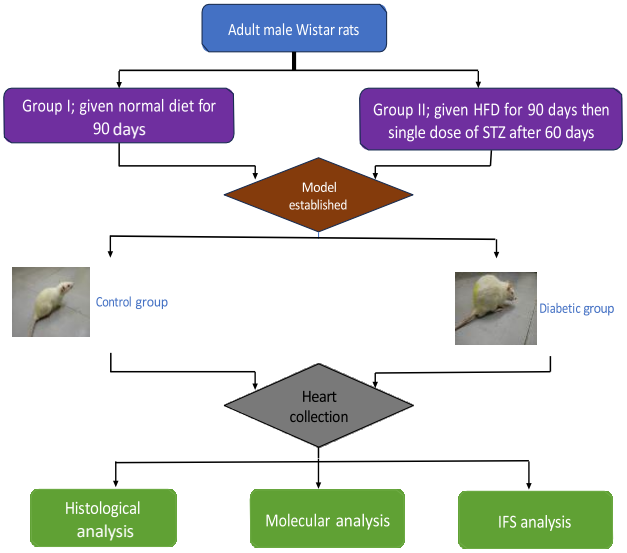
Adult male Wistar rats
Group I; given normal diet for 90 days
Group II; given HFD for 90 days then single dose of STZ after 60 days
Model established
Control group
Diabetic group
Heart collection
Histological analysis
Molecular analysis
IFS analysis

Fig 1. Schematic representation of the modelling

REVIEW OF LITERATURE: -

The development of animal models has played a pivotal role in understanding the pathophysiology of diabetes and its associated complications, including cardiac fibrosis. Diabetic cardiomyopathy, a key contributor to heart failure in diabetic patients, is characterized by myocardial remodelling, hypertrophy, and fibrosis in the absence of overt coronary artery disease. This literature review aims to summarize the current knowledge on the generation of diabetic rat models, methods of characterization, and the detection and assessment of cardiac fibrosis [7].

Introduction to Diabetes and Cardiac Complications: -

Diabetes mellitus (DM), particularly type 1 and type 2, is a systemic metabolic disorder characterized by chronic hyperglycaemia. Long-term diabetes leads to various complications, including diabetic cardiomyopathy (DCM), a disorder of the myocardium independent of coronary artery disease or hypertension. One of the hallmark features of DCM is cardiac fibrosis, contributing to myocardial stiffness and heart failure [2].

Rat models are extensively used to mimic human diabetes and investigate cardiac complications. Models include 4 different types - Streptozotocin (STZ)-induced Type 1 Diabetes Models: STZ selectively destroys insulin-producing β-cells in the pancreas, inducing type 1 diabetes, high-Fat Diet + STZ Models: mimic type 2 diabetes by combining insulin resistance (from diet) and partial β-cell destruction (by STZ), genetic models (e.g., Zucker diabetic rats): exhibit obesity and type 2 diabetes spontaneously, STZ-induced models are particularly useful due to reproducibility and similarity in pathophysiological features with human diabetic cardiomyopathy [8].

Characterization of Diabetic Rat Models after induction, the diabetic state is confirmed through blood glucose monitoring (typically >250 mg/dL confirms diabetes), body weight and food/water intake, Insulin levels via ELISA or immunoassays, histological analysis of pancreatic tissue (for β-cell loss).[9]

Cardiac assessment includes many other ways for better understand of the disease, like- echocardiography for cardiac function, histological and molecular analyses for fibrosis and hypertrophy, measurement of oxidative stress markers and inflammatory cytokines [9].

Mechanisms of Cardiac Fibrosis in Diabetes is also needed to be understood. Cardiac fibrosis is characterized by excessive deposition of extracellular matrix (ECM), particularly collagen types I and III. Mechanisms include a.) hyperglycaemia-Induced Oxidative Stress: Generates ROS that activates pro-fibrotic signalling, b.) inflammation: Elevated TNF-α, IL-6, and MCP-1 promote fibroblast activation, c.) RAAS Activation: Angiotensin II promotes TGF-β1 expression, a central regulator of fibrosis [10].

Detection and quantification of Cardiac Fibrosis can be done by several techniques. Some techniques are used to detect cardiac fibrosis in diabetic rat models. Those are- a. Histological Staining: Masson's Trichrome and Picrosirius Red Staining are standard methods for collagen visualization, b. Molecular Techniques: qPCR and Western Blotting for fibrotic markers- TGFβ1, CTGF, collagen I/III, fibronectin, immunohistochemistry to localize and quantify ECM proteins, c. Imaging Techniques: echocardiography and MRI can assess structural and functional changes, though less commonly used in rats due to cost [11].

After those procedures therapeutic studies and biomarker evaluation should be done as these things will help in understanding of further studies.

Many studies utilize diabetic rat models to test antifibrotic therapies, including- ACE inhibitors/ARBs, antioxidants (e.g., N-acetylcysteine, resveratrol), anti-inflammatory agents (e.g., curcumin, metformin). Therapeutic efficacy is evaluated by reductions in collagen content, TGF-β1 levels, and improvements in cardiac function [1].

Diabetic rat models are instrumental in evaluating antifibrotic agents such as: ACE inhibitors and ARBs: Inhibit R A A S and reduce collagen deposition, Antioxidants (e.g., resveratrol, N-acetylcysteine): Reduce oxidative stress and TGF-β1 activation, anti-inflammatory agents (e.g., curcumin, metformin): Modulate cytokine levels and fibrotic signalling [12][13][14].

MATERIALS AND METHODS: -

● Animals

Species: Male Wistar rats

Age: 8–10 weeks

Weight: 160–190g

Housing: Standard laboratory conditions (22 ± 2°C, 12 h light/dark cycle) with ad libitum access to food and water.

Ethical approval: All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) and performed according to relevant guidelines of the Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA).

● Induction of Diabetes

2.1 Chemical Induction Method (Streptozotocin - STZ)

Reagent: Streptozotocin (STZ, Sigma-Aldrich)

Preparation: Freshly dissolved in cold 0.1 M citrate buffer (pH 4.5)

Dose: 35 mg/kg body weight

Route: Intraperitoneal (i.p.) injection

Control Group: Injected with citrate buffer only

2.2 Confirmation of Diabetes

Method: Tail vein blood sampling using a glucometer

Time: 72 hours post-injection

Criteria: Rats with fasting blood glucose levels >250 mg/dL were considered diabetic.

● Experimental Design

We have divided those rats in 2 different groups->

Groups:

Group I: Control (non-diabetic)

Group II: Diabetic (STZ-induced)

Duration: 3 months post-induction, depending on the objective (fibrosis typically develops in chronic stages).

● Tissue Collection

Procedure: Rats were anesthetized (e.g., with chloroform), and hearts were excised, washed in iced cold PBS.

Tissue: Whole heart for histology and molecular analysis

Storage: In Eppendorf centrifuge tube we kept it in -80°C refrigerator

For histology: Fixed in 4% PFA.

For RNA/protein: Snap-frozen in liquid nitrogen and stored at -80°C

● Histological Analysis of Cardiac Fibrosis

6.1 **Hematoxylin and Eosin (H&E) Staining**

Purpose: General tissue architecture

Protocol:

1. Deparaffinized the slides with 3 changes of xylene, each for 10 minutes.
2. Then cleared the slides with starting from absolute alcohol (100% ethanol) to 90%, 70%, 50%, 30% alcohol gradually. Each for 2 minutes.
3. Then kept those slides in distilled water for 5 minutes.

4. Next, we added 3 to 4 drops of ammonia solution in ~500mL tap water. Then we added Hematoxylin on those slides where the tissues are fixed, kept for 30 secs, and then moved those slides in that beaker where the ammonia and water mixed solution is there.
5. Placed that beaker under running tap water to let the stain develop nicely. As Hematoxylin has a potassium salt in it, that reacts with ammonia of the tap water and stains the nucleus nicely.
6. After a few minutes, I took out those slides and dipped them into 50% ethanol. Then gradually kept on cleaning with 70%, 90% alcohols for 2 minutes.
7. Next, we added Eosin to the 100% alcohol's Coplin jar and added those stained slides into it. Kept for 15 seconds.
8. After taking them out, dipped them into 2 changes of xylene Coplin jars for 5 minutes each.
9. Cleared the slides and mounted them with DPX mounting media, covered them with cover slip.

Result: - Hematoxylin stains the nucleus blue/purple, while Eosin stains the cytoplasm and extracellular matrix pink/red.

6.2 **Masson's Trichrome Staining**

Purpose: Visualization of collagen deposition

Protocol:

1. Deparaffinized the slides with 3 changes of xylene, each for 10 minutes.
2. Then cleared the slides with starting from absolute alcohol (100% ethanol) to 90%, 70%, 50%, 30% alcohol gradually. Each for 2 minutes.
3. Then kept those slides in distilled water for 5 minutes.
4. Celestine blue was added to those slides for 5 minutes.
5. Rinsed the stain with distilled water.
6. Added Hematoxylin to those slides for 30 seconds.

7. Then kept those slides into ammonia (3-4 drops) in ~500mL tap water mixture in a beaker under running tap water for 5 minutes to develop the stain and stain the nuclei.
8. Took out the slides and washed them with 1% acid alcohol for 30 seconds.
9. Then again washed well in distilled water.
10. After that cleaning slides and acid fuschin solution was added onto the slides for 5 minutes.
11. Rinsed the stain with distilled water.
12. Phosphomolybdic acid solution was added to slides for 12 seconds.
13. Then I drained out the solution and pat dry them with tissue paper
14. Methyl blue stain was then added to the slides for 5 minutes.
15. After that, wash them with distilled water.
16. 1% acetic acid wash was done to the slides to destain the extra stains for 15 seconds.
17. Then cleaned the slides with alcohol gradients of 70% to 90% and then to 100% alcohol for 2 minutes each.
18. Then the slides were cleared with 2 changes of xylene for 5 minutes each.
19. After that mounted the slides with DPX mounting media and covered them with cover slip.

Interpretation: Collagen appears blue; myocytes appear red.

6.3 **Van Gieson Staining**

Purpose: This stain is used to differentiate between collagen and smooth muscle in tumours and to demonstrate the increase or decrease of collagen in diseases.

Protocol:

1. Deparaffinized the slides with 3 changes of xylene, each for 10 minutes.

2. Then cleared the slides with starting from absolute alcohol (100% ethanol) to 90%, 70%, 50%, 30% alcohol gradually. Each for 2 minutes.
3. Then kept those slides in distilled water for 5 minutes.
4. Celestine blue was added to the slides for 5 minutes. It stains the nuclei.
5. Rinsed in distilled water.
6. Then stain those slides in Hematoxylin for 5 minutes.
7. Let the stain develop for 5 minutes under running tap water in a beaker full of tap water with a mixture of 3-4 drops of ammonia.
8. Then cleaned the slides with tissue paper, then flooded with Curtis stain for 5 minutes.
9. After that simply just blotted the stain and started dehydrating with direct 70% alcohol then to 90% and 100% alcohol with 2 minutes each.
10. Cleared the slides in 2 changes of xylene for 5 minutes.
11. Mounted them with DPX mounting media and covered them with coverslip.

Results: - Nuclei appears blue, collagen appears as bright red and cytoplasm, muscle cells, fibrin, RBC as yellow.

● Immunohistochemistry / Immunofluorescence

Markers:

α-SMA (alpha smooth muscle actin)

Purpose: Identification of fibrotic pathways and activated fibroblasts

Protocol:

1. Deparaffinized the slides with 3 changes of xylene, each for 10 minutes.
2. Then cleared the slides with starting from absolute alcohol (100% ethanol) to 90%, 70%, 50%, 30% alcohol gradually. Each for 2 minutes.

3. Then kept those slides in distilled water for 5 minutes.
4. Set the Water bath at 85°C beforehand while cleaning.
5. After cleaning procedures, kept those slides samples in a Coplin jar full of antigen retrieval solution [antigen retrieval solution is made of Sodium citrate 1.956 gm and Tween 20 0.5 mL dissolved in Milli Q water, pH was adjusted to 6 for 1 L of the solution.].
6. Kept that Coplin jar in the water bath for 25 minutes at 85°C.
7. Then took out the Coplin jar, left it for cooling at room temperature.
8. After cooling down, the slides were washed in PBS for 2 minutes.
9. After that, blocking was added to the slides for 1 hour. Blocking was made of 2% BSA, 0.1% Triton X-100 which were dissolved in 10 mL of PBS.
10. Then after this step we cleaned the slides and added primary antibodies to those fixed tissues on the slides, which are specific for a marker and kept the slides in a box at 4°C overnight in the dark.
11. Next day, after taking out the slides, discarded the primary antibody. Then dipped those slides into a Coplin jar which had PBST in it. 2 changes of PBST were done to remove the non-specific or lose bindings and then 1 PBS wash was given. Each of those washes was for 5 minutes [PBST was made of 0.2% Triton X-100, 0.1% Tween 20 and 100 mL PBS].
12. After that step of cleaning the slides were flooded with secondary antibodies specific to the primary antibody and kept them in the dark for 2 hours.
13. Then again, those slides were washed and cleaned with 2 changes of PBST and one change of PBS, each for 5 minutes.
14. Then DAPI was added to the slides to stain the nuclei of cells for 15 minutes.
15. Again, those slides were washed with 2 changes of PBST and one change of PBS.
16. Then those were mounted with mounting media and covered with coverslip.
17. Then the slides were seen under a fluorescence microscope.

● Molecular Analysis

8.1 **RNA Isolation**

Purpose: To get the total transcriptome and understand the gene expression better.

Protocol:

1. Dissected the tissue from the heart.
2. Broke the tissue by using a surgical blade into small pieces and finely chopped them.
3. Took 100 µg of that sample.
4. Homogenized it by adding 500 µL of Trizol and left it at room temperature for 5 minutes after that.
5. Added 100 µL of chloroform to it.
6. Shook those centrifuge tubes for 15 seconds in up and down motion vigorously.
7. Put them on an ice bucket for 5 minutes at room temperature.
8. Centrifuge those tubes at 12000 rcf for 15 minutes at 4°C.
9. Kept the supernatant after the centrifugation and discarded the pellet. Put them in new Centrifuge tubes.
10. Precipitated the RNA using 250 µL of isopropanol.
11. Incubated it at -80°C for 1 hour if available or else at -20°C for 2 hours.
12. Again, Centrifuge those tubes at 12000 rcf for 10 minutes at 4°C.
13. Removed the supernatant this time and collected the pellet carefully.
14. Added 75% ethanol of 500 µL to the pellet.
15. Centrifuge them again at 7500 rcf at 4°C for 5 minutes.
16. Then discarded the ethanol carefully, air dried the pellet.
17. Resuspended those pellets in distilled water of 25 µL.

18. Stored them in a -20°C refrigerator.

8.2 **cDNA Preparation**

Reagents supplied:

- Super RTeasy Enzyme mix: 100 µL
- 2x Reaction mix (contains dNTPs, Oligo dT and Random Hexamer): 500 µL
- Nucleus free water: 500 µL

This Super RTeasy enzyme is pre blended with RNase inhibitor.

Storage instruction-> -20°C.

2X reaction mix- 10 µL

Control sample- 10.8577 µL

STZ sample- 16.9491 µL

Step-1: Incubate at 25°C for 5 minutes.

Step-2: Add 2 µL of Super RTeasy enzyme mix to each reaction tube.

Step-3: Incubate at 46°C for 20 minutes.

Step-4: Inactivated the reaction by heating at 95°C for 1 minute.

Step-5: In case busy, keep it at infinity hold.

Purpose: To convert RNA to stable form cDNA for various molecular biology usages.

8.3 **Quantitative Polymerase Chain Reaction (qPCR)**

Gene of interest: MMP9

Housekeeping gene: β-ACTIN

Reagents Needed:

SyBr green- 70 µL

DEPC water- 44.8 µL

Forward primer- 5.6 µL

Reverse primer- 5.6 µL

Sample of cDNA in each well- 1 µL

Protocol:

1. Made two different master mixes in 2 different centrifuge tubes. One for housekeeping gene, another for gene of interest in a dark environment as the SyBr green is light sensitive.
2. Took 9 µL of each master mix in 2 different rows of 96 well plates.
3. Added 1 µL of cDNA samples in each well of two different rows.
4. Made 3 biological replicates of those 2 genes as that made 12 wells full of sample and master mix.
5. Covered the plate carefully with Well Pressure Optical Plate Sealing Membrane.
6. Set the computer and software after that to place the plate in the qPCR machine.
7. Set the running protocol (Denaturation at 95°C initially 15 minutes, again denaturation at 95°C for 15 seconds, annealing at 55.4°C for 12 seconds, then extension at 72°C).
8. Close the machine and let the experiment run.

Purpose: to quantify the amount of DNA or RNA in a sample during the amplification process.

8.4 **Protein Isolation**

Preparation of RIPA Lysis Buffer:

Reagents	Volume (for 20 mL)	Final concentration
5M NaCl	600 µL	Final concentration 150 mM
1 M Tris (pH 8)	1 mL	50 mM

NP- 40	200 µL	1%
10% DOC	1 mL	0.5%
10% SDS	200 µL	0.1%
0.5 M EDTA	200 µL	5mM
Distilled water	16.8 mL	–

Table no. 1 reagent concentrations for lysis buffer

Protocol:

1. Measure the weight of tissue sample.
2. Add 550 µL of PBS and spin at 14000 rcf for 4 minutes at 4°C.
3. After centrifugation discard the PBS.
4. Add RIPA lysis buffer and homogenize the tissue. Add lysis buffer 5 times the weight of heart tissue.
5. Before homogenization add protease and phosphatase Inhibitors.
6. Give heat and cold shock 2-3 times (-20°C for 1 hour, 4°C for 5 minutes).
7. Vortex for 10 seconds. Then proceed for centrifugation at 14000 rcf for 20 minutes at 4°C.
8. Take the supernatant to another centrifuge tube.
9. Store it in -80°C refrigerator.

Purpose: to measure the overall amount of protein in a sample. Elevated protein levels may indicate inflammation or infection, while low levels can suggest kidney problems or malnutrition.

RESULTS and DISCUSSION: -

After all the procedures and methods were done

● **Tissue Collection**-

The diabetic rat model was successfully established through streptozotocin (STZ) induction, as confirmed by sustained hyperglycaemia (>250 mg/dL) in treated animals compared to controls. Over a period of 8-12 weeks, diabetic rats exhibited significant weight loss, polydipsia, and polyuria- clinical hallmarks of diabetes mellitus.

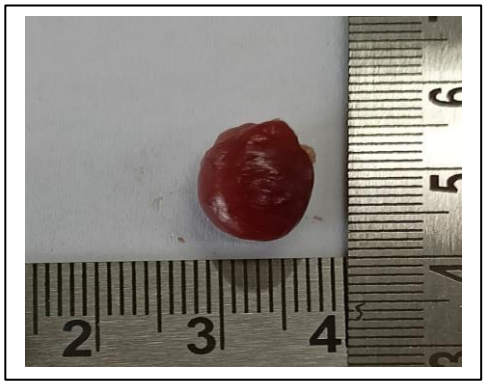

Fig 2.A:
CONTROL

Fig 2.B:
STZ INDUCED DIABETIC HEART

Fig 2. Tissue sample collection after sacrificing the rat models

● **Confirmation of diabetes**-

Now from few data of body weight and blood glucose concentration, heart weight to body weight ratio we confirmed the diabetes was successfully induced. Where, fig2.A is showing the increase in body weight with time then after certain period it falls again because of diabetes, fig 2.B is showing the increase in concentration of blood glucose due to STZ induced diabetes. There the 2.C figure is showing the heart weight to body weight to body weight ratio of two different groups of rat models.

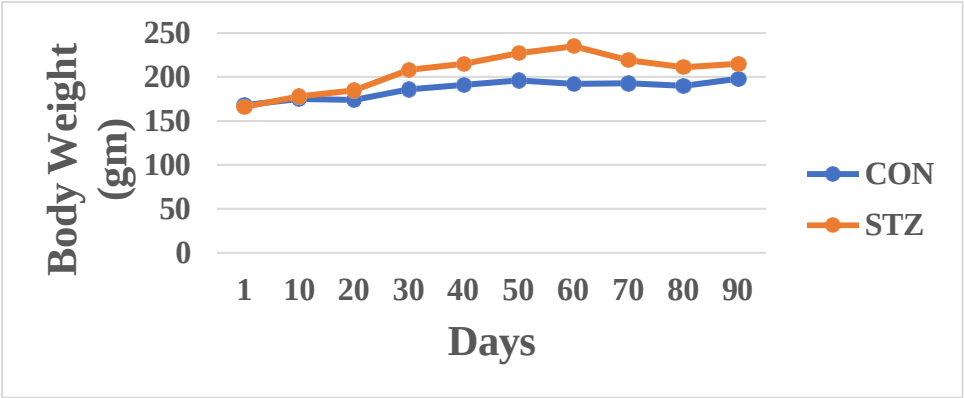

Fig 3.A

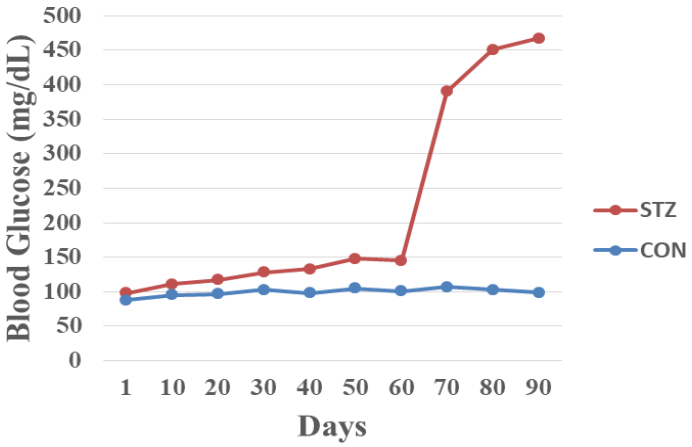

Fig 3.B

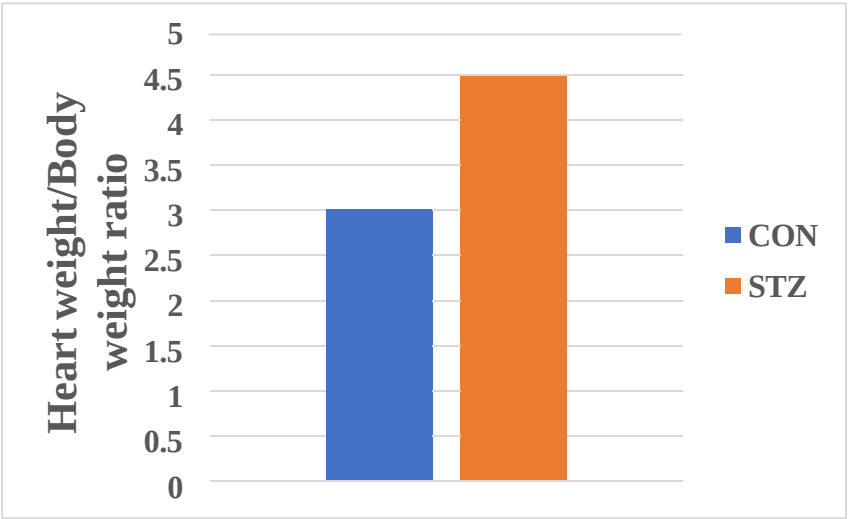

Fig 3.C

Fig 3. Graphs of- 3.A Body weight changes in two different group of rats, 3.B Blood glucose concentration changes in different groups of rat models, 3.C Heart weight to body weight ratio.

● **Histological Analysis**-

H&E Staining:

Histological examination of cardiac tissue using Hematoxylin and Eosin (H&E) staining revealed notable morphological differences between control and diabetic rat hearts. In control (non-diabetic) rats, myocardial architecture was well-organized with clear striations and tightly packed cardiomyocytes exhibiting normal nuclei and minimal interstitial space.

In contrast, the diabetic rat group demonstrated evidence of myocardial structural disruption. Cardiomyocytes appeared disorganized, with varying degrees of myocyte degeneration and cytoplasmic vacuolization. A mild to moderate increase in interstitial space was observed, suggesting early fibrotic changes. Additionally, infiltration of mononuclear inflammatory cells was noted in the interstitial regions, consistent with inflammation-induced remodelling.

While H&E staining is not specific for collagen detection, the expansion of interstitial and perivascular spaces coupled with cellular disarray suggests the presence of fibrosis-related changes, corroborating with the known pathological features of diabetic cardiomyopathy.

The findings from H&E staining indicate significant structural and cellular alterations in the myocardium of diabetic rats, supporting the successful establishment of a diabetic model. The disarray of cardiomyocytes, interstitial widening, and inflammatory infiltration are hallmarks of early cardiac remodelling and precede overt fibrosis.

H&E staining, while nonspecific for fibrosis, remains a valuable tool for detecting overall histopathological damage. The myocardial alterations observed here suggest a pro-fibrotic environment, likely driven by chronic hyperglycaemia, oxidative stress, and inflammation. These changes align with previously reported features of diabetic cardiomyopathy in rodent models and human studies.

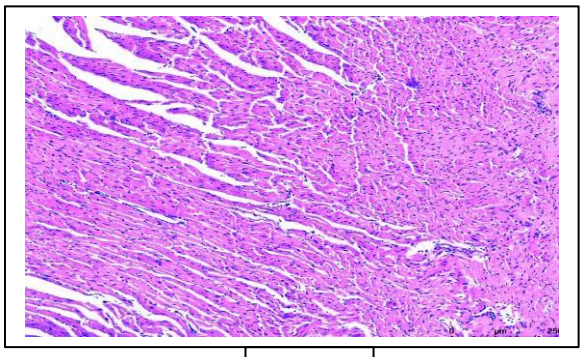

Fig 4.A

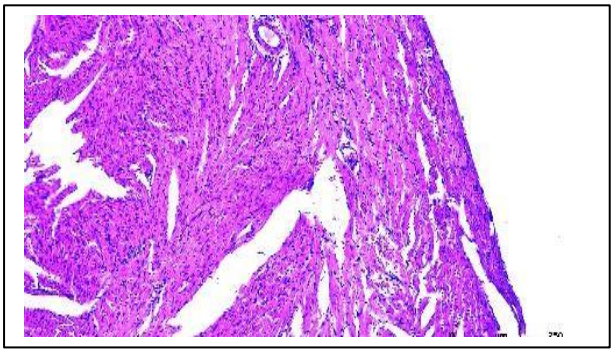

Fig 4.B

Fig 4. Histological analysis by H&E Staining. 4.A Control sample tissue's microscopic image, 4.B STZ induced diabetic heart tissue's microscopic image.

Masson's Trichrome Staining:

Masson's Trichrome staining was performed to evaluate the extent of cardiac fibrosis in control and diabetic rat heart tissues. In control (non-diabetic) myocardial sections, normal cardiac architecture was preserved with minimal collagen deposition, as evidenced by the light blue staining of interstitial regions.

In contrast, myocardial tissues from diabetic rats showed a marked increase in collagen deposition, particularly in the interstitial and perivascular regions, characterized by intense blue staining. The fibrotic areas were significantly more prominent in the diabetic groups compared to controls, indicating excessive extracellular matrix (ECM) accumulation.

The results from Masson's Trichrome staining clearly demonstrate the presence of cardiac fibrosis in the diabetic rat model. The observed increase in collagen deposition is a hallmark of myocardial remodelling and supports the hypothesis that chronic hyperglycaemia contributes to structural alterations in cardiac tissue.

These findings align with previous studies suggesting that diabetic cardiomyopathy is characterized by increased ECM accumulation, leading to myocardial stiffness, impaired cardiac function, and eventual heart failure. The perivascular and interstitial fibrosis observed may be driven by hyperglycaemia-induced oxidative stress, activation of profibrotic cytokines such as TGF-β, and enhanced fibroblast activity.

The significant difference in fibrotic area between diabetic and control groups validates the effectiveness of the diabetes induction protocol and supports the use of this model for studying diabetic cardiac complications.

Fig 5.A

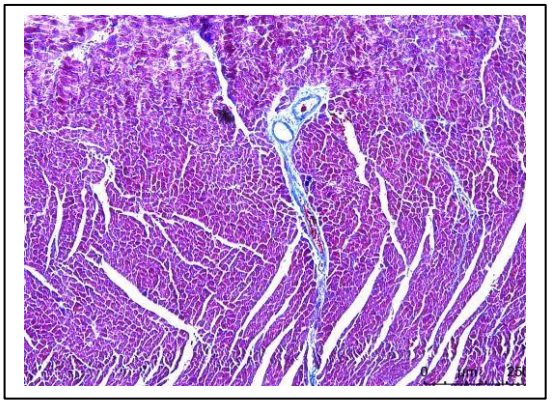

Fig 5.B

Fig 5. Histological analysis by Masson's Trichrome staining. 5.A is showing the control sample and 5.B is showing the increase collagen deposition in STZ induced diabetic heart tissue sample.

Van Gieson Staining:

Van Gieson staining of cardiac tissue sections revealed distinct differences in collagen deposition between diabetic and control rat hearts. In control rats, minimal interstitial collagen deposition was observed, with myocardial fibers appearing well-organized and structurally intact. In contrast, cardiac tissues from diabetic rats exhibited extensive collagen accumulation, particularly in the interstitial and perivascular regions.

Collagen fibers were stained bright red, while muscle fibers appeared yellow, allowing clear differentiation and evaluation of fibrosis.

Van Gieson staining effectively demonstrated increased cardiac fibrosis in diabetic rats, confirming the development of diabetic cardiomyopathy characterized by excessive collagen deposition. The prominent red staining in diabetic cardiac tissues indicates enhanced accumulation of type I collagen, which is a hallmark of myocardial fibrosis.

This structural remodelling is likely attributed to chronic hyperglycaemia-induced oxidative stress, inflammation, and activation of fibrogenic pathways such as TGF-β1/Smad signalling. Fibrosis compromises myocardial elasticity and

contributes to diastolic dysfunction, which is commonly observed in diabetic heart disease.

These findings are consistent with previous studies reporting increased myocardial fibrosis in experimental models of type 1 and type 2 diabetes. The observed histopathological changes validate the successful generation of a diabetic rat model and support its utility for further investigation of the mechanisms and therapeutic targets of diabetic cardiomyopathy.

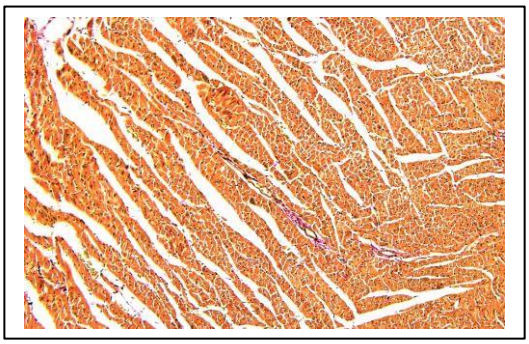

Fig 6.A

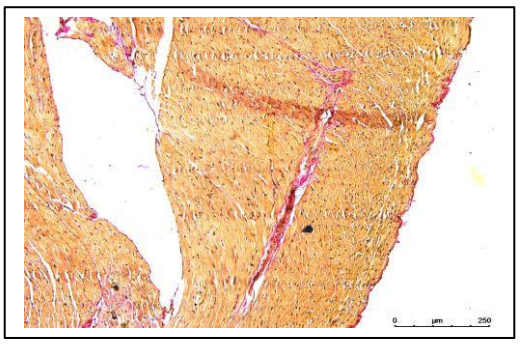

Fig 6.B

Fig 6. Histological analysis by Van Gieson Staining. 6.A is showing the microscopic photo of heart tissue, collected from control group pf rat models and fig 6.B is showing the photo of STZ induced diabetic heart tissue of rat models.

● **Molecular Analysis-**

After doing RNA Isolation, cDNA was made by doing PCR and by running gradient PCR protocol and Agarose Gel Electrophoresis the optimum annealing temperature was set to do the real time PCR for our gene of interest.

Quantitative Polymerase Chain Reaction:

Quantitative real-time PCR (qPCR) analysis revealed a significant upregulation of MMP9 (Matrix Metalloproteinase-9) gene expression in the cardiac tissues of diabetic rats compared to non-diabetic controls. The relative mRNA expression levels of MMP9, normalized to β-actin as the housekeeping gene, showed a 5-fold increase in the diabetic group.

This elevation in MMP9 expression was consistent across all diabetic samples and correlated with the duration and severity of hyperglycaemia. Amplification

plots showed efficient and specific amplification, confirmed by melt-curve analysis showing a single peak for MMP9 and β-actin.

The significant upregulation of MMP9 in diabetic rat hearts suggests an active role of this metalloproteinase in the pathophysiology of diabetic cardiomyopathy and cardiac fibrosis. MMP9 is known to degrade extracellular matrix (ECM) components and facilitate tissue remodelling; its overexpression is often associated with pathological remodelling and fibrosis.

In the context of diabetes, chronic hyperglycaemia induces oxidative stress and inflammatory signalling, which are known stimuli for MMP9 expression. The elevated levels observed in this study align with previous findings that link MMP9 upregulation to enhanced ECM turnover and myocardial fibrosis.

Furthermore, the increased MMP9 expression observed parallels the histopathological findings of myocardial fibrosis, such as interstitial collagen deposition and myocyte disarray, observed in this study's Masson's Trichrome-stained cardiac sections. This reinforces the hypothesis that MMP9 contributes to the degradation-repair imbalance in the myocardial ECM, promoting fibrotic remodelling.

Overall, these findings support the validity of the diabetic rat model in mimicking key molecular and structural features of human diabetic cardiomyopathy and highlight MMP9 as a potential biomarker or therapeutic target in the early detection and treatment of cardiac fibrosis in diabetes.

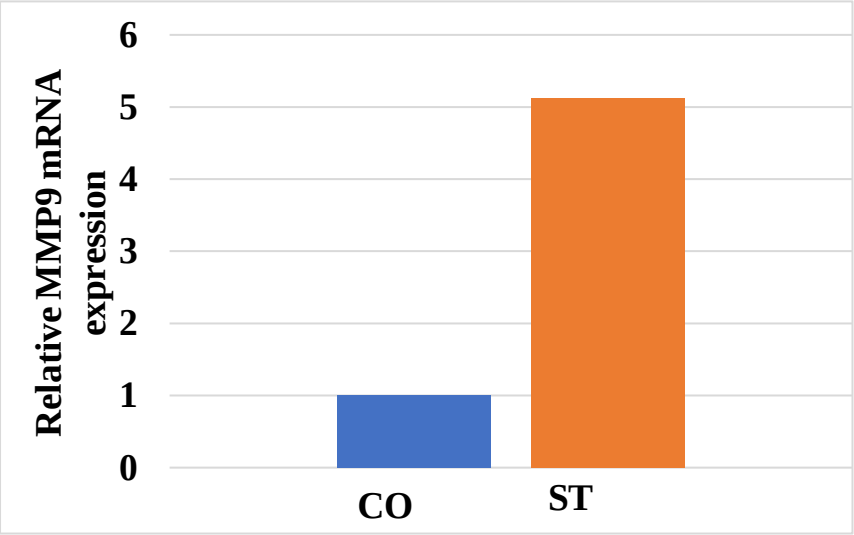

Fig 7. is showing the fold change graph by real time PCR analysis of MMP9 against the housekeeping gene β-actin.

● **Immunofluorescence Analysis-**

Immunofluorescent analysis of α-SMA expression revealed significant upregulation of α-SMA-positive cells in the myocardial tissue of diabetic rats compared to controls. In control hearts, α-SMA staining was primarily limited to vascular smooth muscle cells, as expected. In contrast, the diabetic group showed increased α-SMA immunoreactivity extending into the interstitial regions of the myocardium, indicating the activation of cardiac fibroblasts into myofibroblasts.

Quantitative image analysis confirmed a statistically significant increase in the α-SMA-positive area per field in the diabetic group. These changes were consistent across different heart regions examined, including the left ventricle and interventricular septum. Immunofluorescence microscopy further validated these findings, demonstrating enhanced α-SMA signal intensity and a more diffuse pattern of expression in diabetic hearts.

The upregulation of α-SMA in diabetic rat hearts serves as a hallmark of myofibroblast activation, a key event in the progression of cardiac fibrosis. Myofibroblasts are responsible for the excessive deposition of extracellular matrix proteins, including collagen, which disrupts myocardial architecture and contributes to diastolic dysfunction. The observed elevation in α-SMA expression in diabetic myocardium aligns with the histological evidence of interstitial and perivascular fibrosis reported in earlier sections.

This finding supports the notion that chronic hyperglycaemia in the diabetic model promotes fibroblast-to-myofibroblast differentiation, likely through profibrotic signalling pathways such as TGF-β/Smad and oxidative stress-related mechanisms. The localization of α-SMA outside vascular structures in the diabetic group also suggests an expansion of activated fibroblast populations in response to metabolic and inflammatory stimuli characteristic of diabetic cardiomyopathy.

Our results are consistent with previous studies indicating α-SMA as a reliable marker for early and progressive cardiac fibrosis in diabetic models. These data further validate the successful induction of cardiac fibrotic remodelling in our diabetic rat model and reinforce the utility of α-SMA as a molecular indicator for therapeutic evaluation in future studies.

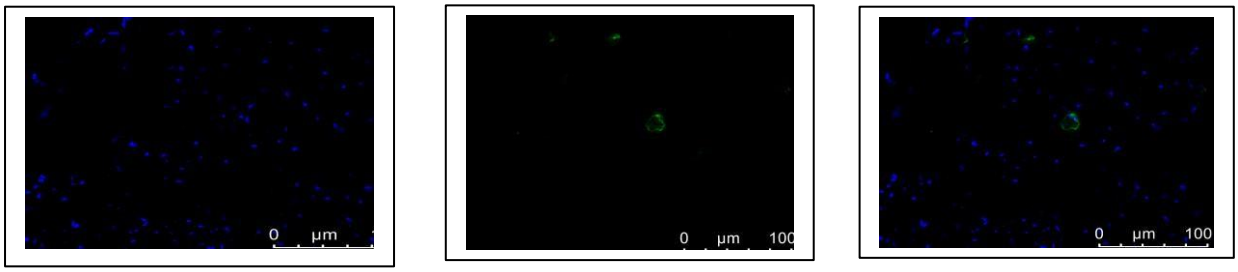

Fig 8. Control tissue sample's IFS microscopic images for DAPI (stains the nuclei of cells) and antibody against α-SMA (green).

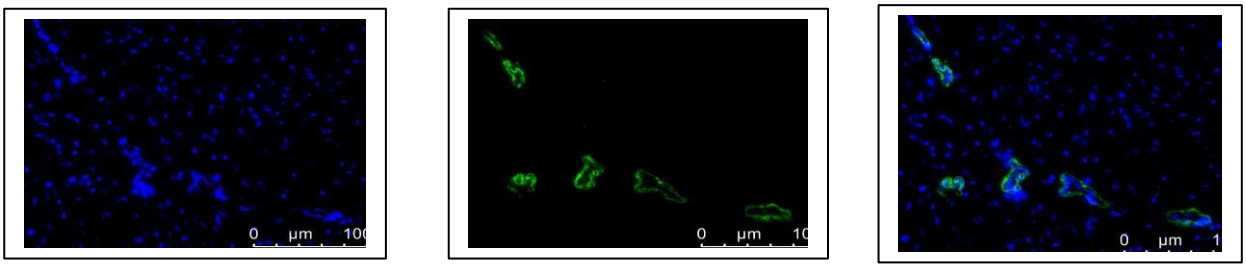

Fig 9. STZ induced diabetic heart tissue sample's IFS microscopic images for DAPI (satins the nuclei of cells) and antibody against α-SMA (green)

CONCLUSION: -

In this study, a diabetic rat model was successfully generated and comprehensively characterized to mimic the pathophysiological conditions observed in human diabetes mellitus. The induction of diabetes led to persistent hyperglycemia, altered metabolic profiles, and systemic complications consistent with the diabetic state. Importantly, histological, and molecular analyses confirmed the development of cardiac fibrosis in adult diabetic rats, indicating progressive myocardial remodeling as a consequence of chronic hyperglycemia. These findings validate the utility of this model for investigating the mechanisms underlying diabetic cardiomyopathy and for evaluating potential therapeutic interventions targeting cardiac fibrosis. Overall, the established model provides a robust platform for future preclinical studies aiming to mitigate cardiovascular complications associated with diabetes.

Appendix: -

- For RIPA lysis buffer the reagents were added-

Reagents	Volume (for 20 mL)	Final concentration
5M NaCl	600 µL	Final concentration 150 mM
1 M Tris (pH 8)	1 mL	50 mM
NP- 40	200 µL	1%
10% DOC	1 mL	0.5%
10% SDS	200 µL	0.1%
0.5 M EDTA	200 µL	5mM
Distilled water	16.8 mL	–

- For blocking of IFS image analysis, before adding primary antibody-

  2% BSA, 0.1% Triton X-100 which were dissolved in 10 mL of PBS.

- For PBST we added-
  0.2% Triton X-100, 0.1% Tween 20 mL and 100 mL PBS.

- For antigen retrieval solution we added-
  Sodium citrate 1.956 gm and Tween 20 0.5 mL dissolved in Milli Q water; pH was adjusted to 6 for 1 L of the solution.

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